Dissemination of *bla*$_{VIM}$ in Greece at the peak of the epidemic of 2005–2006: clonal expansion of *Klebsiella pneumoniae* clonal complex 147

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Abstract

VIM-producing *Klebsiella pneumoniae* (n = 21) isolated from ten Greek hospitals during 2003–2007 were characterized with multilocus sequence typing (MLST), semi-automated repetitive sequence-based PCR (rep-PCR) (Diversilab), plasmid replicon typing, serotyping and screening for multiple resistance determinants. The isolates were selected to represent different strain clusters (defined by >80% similarity) according to pulsed-field gel electrophoresis. MLST identified three major clonal complexes (CCs); CC147 (n = 8), CC18 (n = 5) and CC14 (n = 3). Plasmid replicon typing showed that IncA/C and/or IncFII$_K$ replicons were detected among isolates in each of the major CCs. Good concordance was observed between semi-automated-rep PCR genotyping and MLST.

Keywords: MLST, clonal complex, metallo-β-lactamase, AmpC, plasmid replicon
Original Submission: 26 October 2012; **Revised Submission:** 15 January 2013; **Accepted:** 1 February 2013
Editor: R. Cantón
Article published online: 7 March 2013
Clin Microbiol Infect 2014; **20:** 34–37
10.1111/1469-0691.12187

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Klebsiella pneumoniae harbouring metallo-β–lactamases are a serious threat to the carbapenem class of antimicrobials. Some years after the first detection of VIM in Verona, Italy, in the year 1999, the prevalence of VIM-producing *K. pneumoniae* (VPKP) became widespread in some regions of Southern Europe. VPKP has given rise to major problems both with respect to infection control and treatment [1–4]. In most cases, isolates harbouring this class of enzyme have been isolated from hospitalized patients in intensive care units (ICUs) [4]. Data are still largely lacking regarding the clonal structure of strains disseminating VIM. Therefore, the objective of this study was to determine the clonal diversity of VPKP isolated in various parts of Greece at the peak of the epidemic of 2003–2006 by using multilocus sequence typing (MLST).

After the first report of VPKP in Greece in 2003 [1], all 40 hospitals participating in the Greek System for Surveillance of Antimicrobial Resistance (www.mednet.gr/whonet) forwarded all VPKP clinical isolates to the National School of Public Health in Athens for further study. Isolates were from human sources, mainly from blood, wound, urinary and respiratory tract samples. Molecular fingerprints for all isolates were prepared by pulsed-field gel electrophoresis (PFGE) with *Xba1* restriction and analysed in GelCompar (Applied Maths, SintMaartens-Latem, Belgium). Fingerprints were classified as distinct when similarity was <80%.

The present study included 21 isolates representing important and distinct PFGE-types. Eleven isolates (52.3%) were derived from five different hospitals (I-V) located in Athens, while the rest of the isolates were collected from another five different hospitals (VI-X) belonging to four different Greek cities (Table 1). A PCR-based serotyping method was conducted to determine the capsular types K1, K2, K5, K20, K54 and K57, and screening for the virulence genes *allS*, *rmpA* and *wcaG* was performed by PCR amplification. [5], while PCR-based replicon typing of plasmids was performed according to Carattoli *et al.* [6]. MLST was performed by PCR amplification and sequencing of seven conserved housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB* and *tonB*) (http://www.pasteur.fr/genopole/PF8/mlst/

TABLE 1. Characteristics of 21 VIM-producing *K. pneumoniae* strains isolated from Greece

Strain	Hospital	Area	Specimen	ST	CC	DL type	PBRT	Serotype[a]	Virulence gene	CTX-M	PMQR[b]	16S[c]	pAmpC	Isolation year
VPKP194	I	Athens	Unknown	147	147	B	ND	-	-	-	-	-	-	-
VPKP203	II	Athens	Urine	147	147	B	A/C	-	-	-	*aac(6')-Ib-cr*	-	-	2006
VPKP213	V	Athens	Sputum	147	147	B	ND	-	-	-	-	-	-	2005
VPKP842	VII	Thessaloniki	Wound	147	147	B	ND	-	-	-	-	-	MOX	2007
VPKP18	IX	Crete	Wound	147	147	B	FIIK	-	-	-	-	-	-	2005
VPKP33	IX	Crete	Urine	147	147	B	FIIK	-	-	-	*aac(6')-Ib-cr*	-	-	2005
VPKP267	VI	Thessaloniki	Blood	675	147	B	ND	-	-	-	-	-	-	2006
VPKP754	X	NW Greece	Unknown	677	147	B	A/C, FIIK	-	-	-	-	-	-	2007
VPKP205	II	Athens	Blood	17	18	F	FIIK	-	-	-	*aac(6')-Ib-cr*	-	-	2006
VPKP440	III	Athens	Blood	17	18	F	FIIK	-	-	-	-	-	-	2006
VPKP309	VIII	Piraeus	Sputum	17	18	F	FIIK	-	-	-	*aac(6')-Ib-cr*	-	-	2006
VPKP284	VI	Thessaloniki	Blood	676	18	E	A/C, FIIK	-	-	-	-	-	-	2006
VPKP307	VIII	Piraeus	Blood	676	18	E	A/C, FIIK	-	-	-	*aac(6')-Ib-cr*	-	MOX	2006
VPKP374	IV	Athens	Sputum	14	14	C	A/C, FIIK	K2	-	-	*aac(6')-Ib-cr*	-	-	2006
VPKP430	III	Athens	Wound	14	14	D	A/C, FIIK	K2	-	-	*aac(6')-Ib-cr*	-	-	2003
VPKP306	VIII	Piraeus	Catheter	627	14	C	A/C, FIIK	-	-	-	-	-	-	2006
VPKP389	IV	Athens	Blood	36	36	A	FIIK	-	-	-	-	-	-	2006
VPKP6	I	Athens	Unknown	36	36	A	A/C, FIIK	-	-	-	*qnrS*	-	-	2005
VPKP220	V	Athens	Pus	706	-	B	FIIK	-	-	-	-	-	-	2006
VPKP229	V	Athens	Unknown	674	-	G	A/C, N	-	-	-	*aac(6')-Ib-cr*	-	-	2006
VPKP83	X	NW Greece	Blood	323	26	H	A/C	-	-	-	*aac(6')-Ib-cr*	-	-	2005

ND, not detected.
[a]The following serotypes were sought by PCR: K1, K2, K5, K20, K54 and K57.
[b]The following genes encoding PMQR (plasmid-mediated quinolone resistance) were sought: *qnrA*, *qnrB*, *qnrC*, *qnrS* and *aac (6')-Ib-cr*.
[c]The following 16S rRNA methylases were screened for: *armA*, *rmtA*, *rmtB* and *rmtD*.

Kpneumoniae.htm). Based on allelic profiles, a graphical tool-based minimal spanning tree (MST) built in Bionumerics 6.0 (Applied-Maths) was constructed. The genetic relatedness of isolates was also evaluated with DiversiLab (DL), a semi-automated repetitive-sequence-PCR (rep-PCR)-based microbial typing system (bioMérieux, Marcy l'Etoile, France). Isolates were assigned to the same DL type if they had >93% similarity and $\leq$2 peak differences in the electropherogram overlay analysis [7]. Additionally, PCR-based screening methods were carried out to determine the concomitant carriage of extended-spectrum, β-lactamase genes (*bla*$_{CTX-M}$) [8], plasmid-mediated quinolone resistance genes (*qnrA, qnrB, qnrC, qnrS, aac-(6')-Ib-cr*) [9,10], 16S rRNA methylase genes (*armA, rmtB, rmtD*) [11] and plasmid-mediated *AmpC* β-lactamase genes (CIT, MOX, FOX, ACC, EBC and DHA) [12].

The population structure of 21 VPKP isolates is shown in Fig. 1 with 11 sequence types (STs) classifiable. The most frequently detected sequence type was ST147, with six representative isolates. Additionally, two SLVs of this ST were detected (ST675 and ST677), making the total number of isolates in clonal complex (CC) 147 8/21 (38%). Isolates belonging to clonal lineage CC147 were detected in all regions except Piraeus, and have also been described in previous strain imports from Greece to Scandinavia [13]. Another sequence type, ST17, had three representative isolates, and together with its SLV ST674 (n = 2), formed the second clonal complex, CC18. The third clonal complex, CC14, was formed between ST14 (n = 2) and ST627 (n = 1), comprising strains from Athens and Piraeus, respectively. Capsular type K2 was detected in the two isolates of ST14, as has also recently been reported for NDM-1-carrying *K. pneumoniae* of ST14 [7]. Other detected sequence types were ST36 (n = 2) and ST323 (n = 1). ST36 did not cluster with any of the clonal complexes, differing from isolates in CC18 by 3 alleles. ST323 differed from CC18 isolates by only two alleles.

PCR-based replicon typing (PBRT) revealed that IncFII$_K$ occurred in the majority of the isolates (n = 15/21), followed by IncA/C (n = 10/21). Isolates belonging to the major clonal complexes or STs hosted both replicon IncFII$_K$ and IncA/C, whereas IncA/C plus IncN was hosted by a single isolate. The IncA/C plasmid type is well known for its role in the dissemination and wide transmission of both NDM-1 and VIM globally [13,14], whereas IncFII$_K$ has been responsible for the acquisition and dispersion of KPC-2 and CTX-M-1 amongst *K. pneumoniae* in Greece [15]. One previous study has shown that the linkage of VIM carbapenemase with importation to Scandinavia from Greece has greatly been facilitated by broad host-range IncA/C and IncN plasmids hosted by sequence type ST36, ST147 and ST323 [13]. It should be noted that the specific relationship between *bla*$_{VIM}$ and plasmid replicons was not sought in this study.

The genetic relatedness among this entire collection of isolates was also assessed by the DiversiLab microbial typing system. The DiversiLab rep-PCR system grouped all the STs into eight DL types (A-H) (Table 1). The DiversiLab system assigned STs belonging to the major clonal lineages to the same DL type, with the exception of one isolate of ST14 that was assigned to a different DL type than the other ST14 isolates. Moreover, STs that did not belong to any of the clonal complexes were also unique according to the DiversiLab system, as has previously been shown in NDM-1 producing